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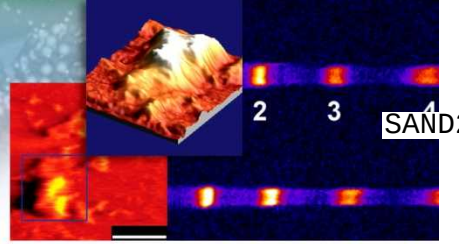

SAND2017-1519C

Quenching of Unincorporated Amplification Signal Reporters (QUASR) for Robust Monitoring of Isothermal DNA and RNA Amplification Assays

Robert Meagher
Sandia National Laboratories
Livermore, CA, USA

Molecular Medicine TriConference
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Livermore, CA

Acknowledgements

Sandia team

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- Aashish Priye – Smart phone platform
- Cameron Ball – Portable devices, Assay characterization
- Sara Bird – Virology
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Collaborators

- Lark Coffey (UC Davis)
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- Scott Weaver (UTMB)
- Barbara Johnson (CDC)
- Brandy Russell (CDC)

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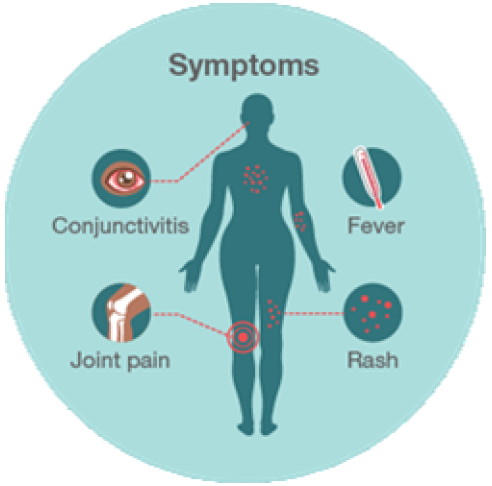
Outline

- Motivation: (RT) LAMP as a technique to enable portable Nucleic Acid Amplification Tests
- QUASR chemistry improves (RT) LAMP detection
- Implementing QUASR for a smart phone-based Zika detection assay

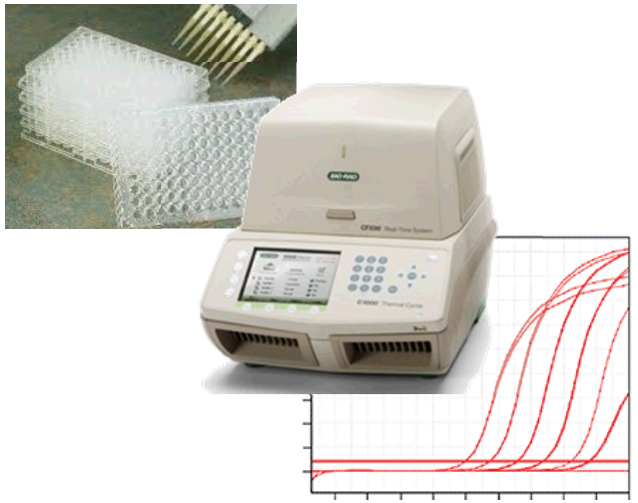
Conventional Laboratory Diagnostics for viral infections

- Diagnostic tests for viral infections include:
  - **Culture** –"gold standard" in many cases, but this is slow, technically challenging, has low sensitivity for many viruses (*e.g.* dengue, Zika) and exceedingly risky for others (*e.g.* Ebola).
  - **Serology** – detecting immune response to viruses; most sensitive late in infection (after seroconversion), but may suffer low specificity (*e.g.* cross-reactive response for flaviviruses)
  - **Nucleic acid detection**: detection of viral RNA; most sensitive early in infection (viremic phase)

- qRT-PCR has great sensitivity and precision but requires a well-equipped laboratory
  - Need to extract RNA (cleanup/concentrate)
  - Reagents require refrigeration
  - The instrumentation is (usually) power hungry and not portable, nor is the rest of the workflow.



www.cdc.gov



Challenges in Deployed Diagnostics

Sample transport: safety, stability, security, and speed?

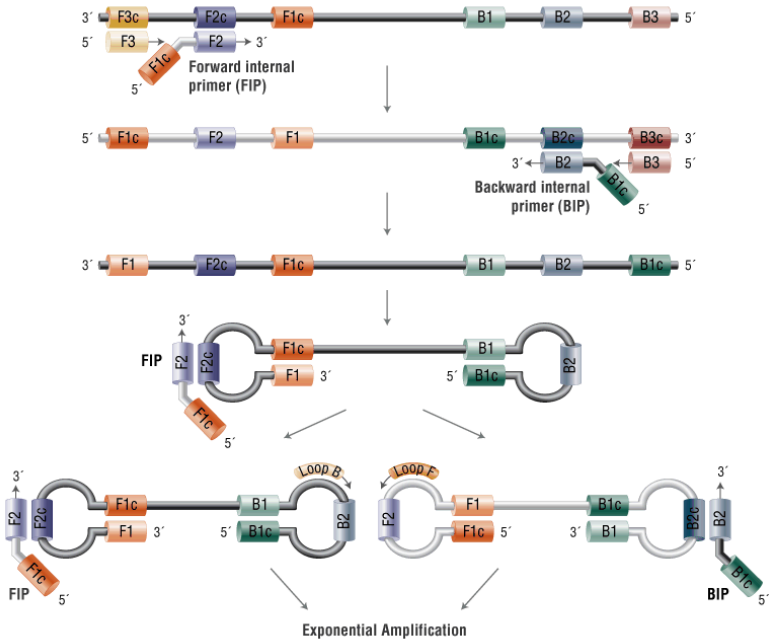
MMWR 2014 / Vol. 63 / No. 50 (Liberia / Ebola outbreak)
Photos: Justin Williams, Sampson Dolo

- Emerging, re-emerging, and neglected diseases like Zika and Ebola occur in parts of the world where medical infrastructure is lacking.
- Can we make a *simple*, *self-contained* diagnostic assay?
- Utilize smart phone capabilities for assay control, scoring, data reporting
- Need robust assay chemistry, cheap consumables, and simple instrumentation.

LAMP is a PCR alternative well suited to low resource settings

- Loop Mediated Isothermal Amplification: primer-based amplification of DNA/RNA targets
- Fast (5-20 min), robust, simple, sensitive
- Low capital expense/Low power
- Can work with minimal/no sample pretreatment

- Can’t easily multiplex
- Most detection techniques are non-specific (turbidity, colorimetric, etc)
- Prone to false positives
- Less quantitative than qPCR

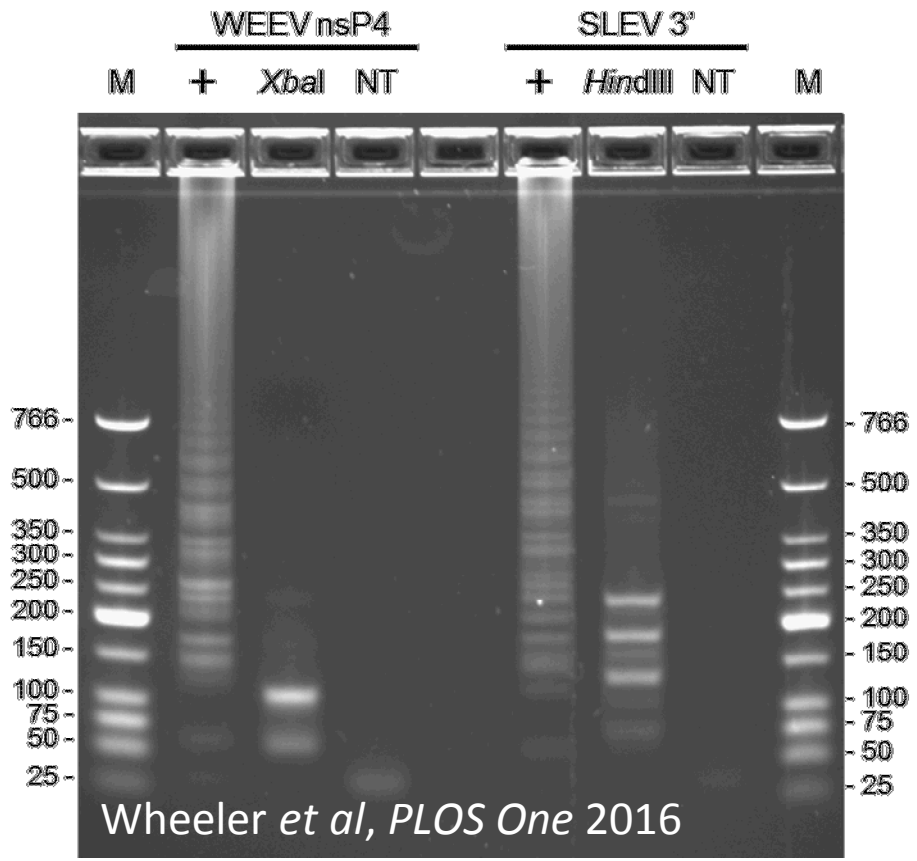


www.neb.com

Complex reaction scheme involves strand displacement instead of thermal denaturation

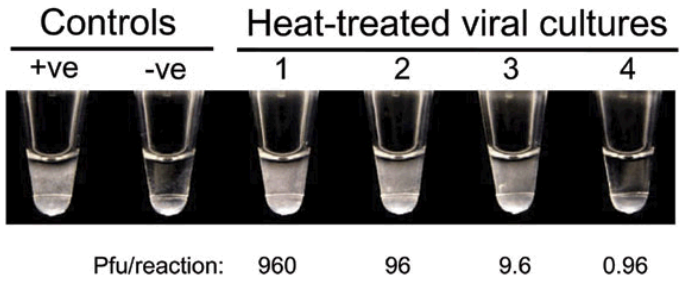
How to know if LAMP worked? (old school)

A. Run product on a gel, with optional target-specific restriction digest



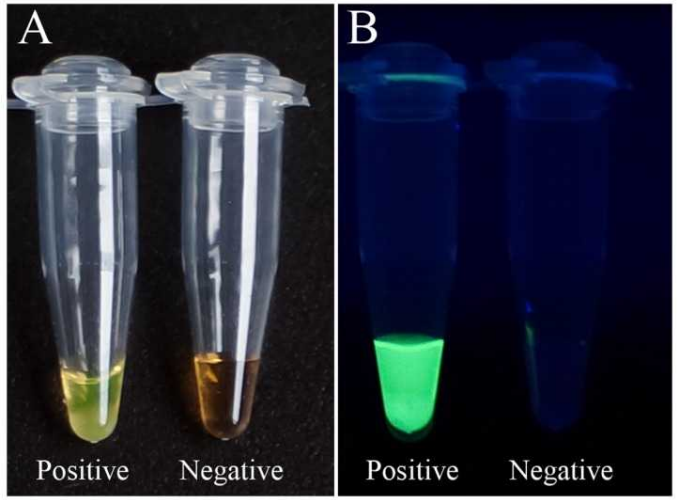
Wheeler *et al*, *PLOS One* 2016

B. Turbidity (precipitation of Mg pyro-phosphate, from making a ton of DNA)



Jayawardena, *Emerg. Inf. Dis.* 2007

C. Post-reaction, open the tube and add a ton of SYBR Green

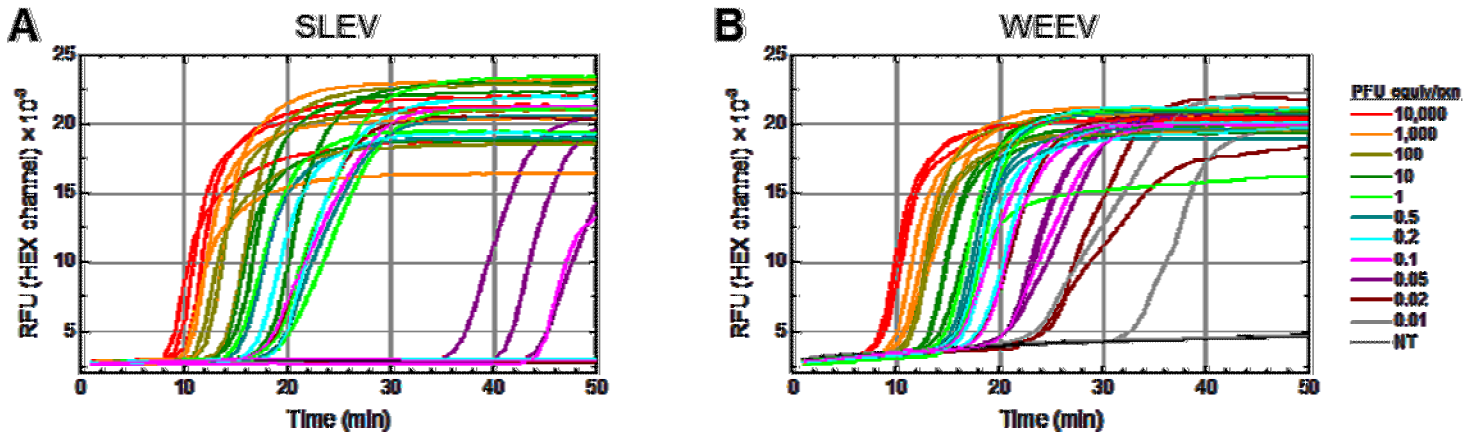


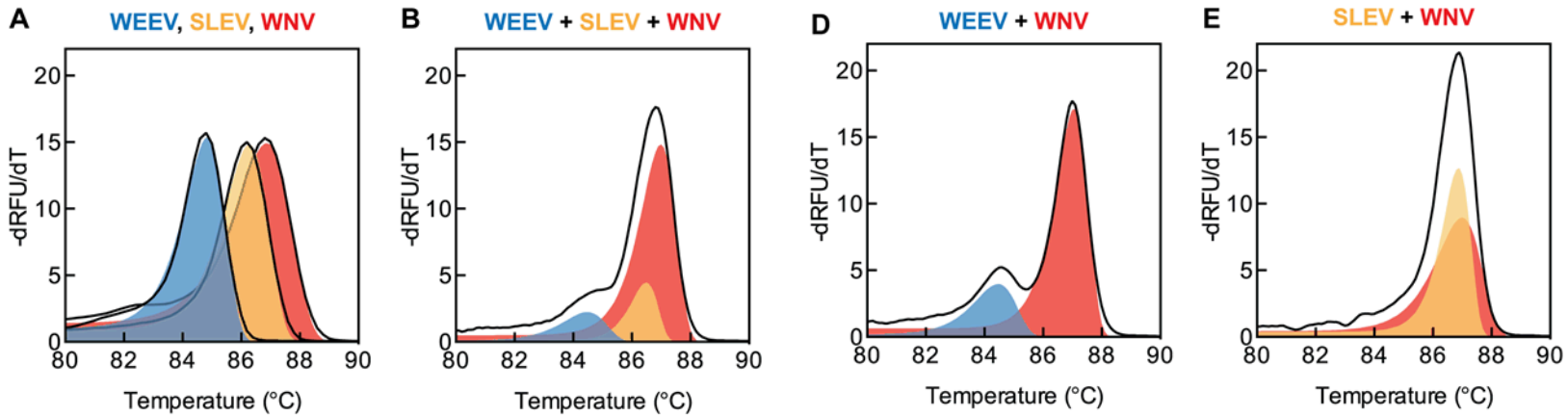
Nie *PLoS One* 2012

D, E, F, G... Other nonspecific indicators of total DNA synthesis…

How to know if LAMP worked? (new school)

- Real-time monitoring with LAMP-compatible intercalating dye (*e.g.* SYTO 82)
- High resolution melt curves (capable of multiplexing)

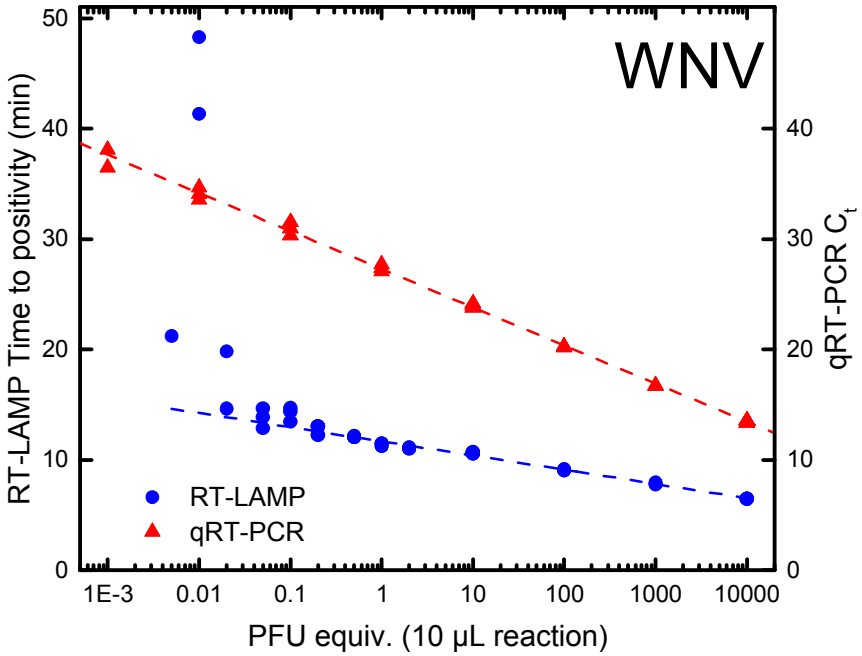


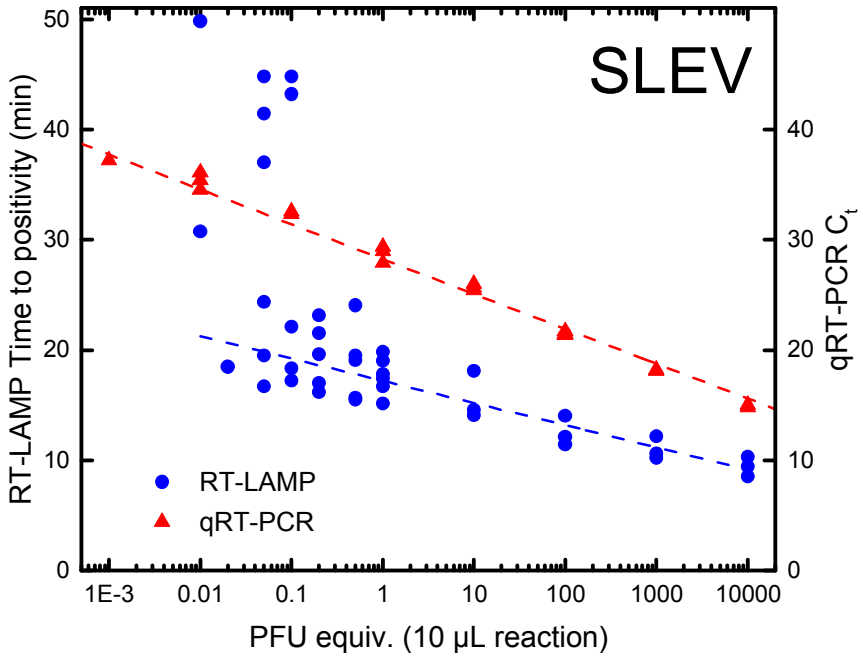


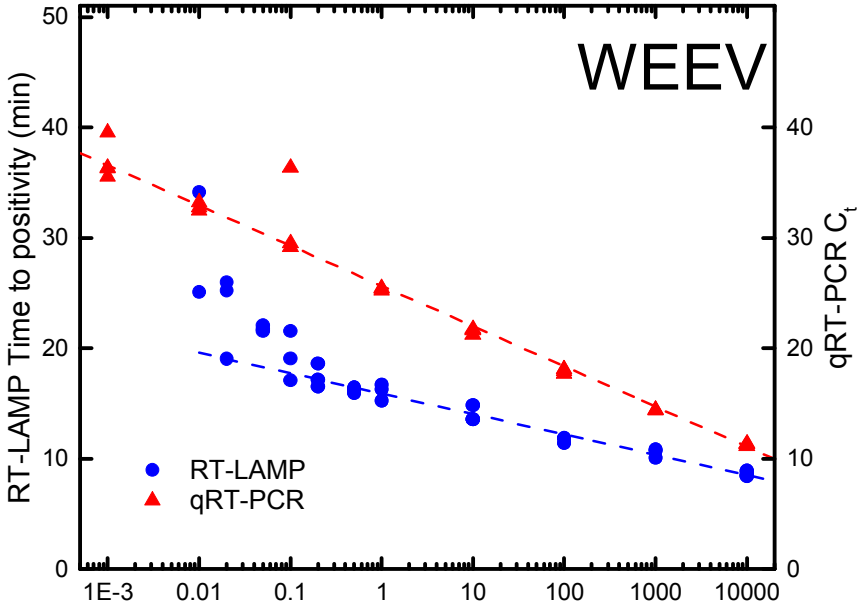
Wheeler *et al*, *PLOS One* 2016

RT-LAMP *vs* qRT-PCR

- In all cases tried, qRT-PCR is ~1 log more sensitive than RT-LAMP
- RT-LAMP for WNV, WEEV, SLEV all detects down to 0.01 PFU equiv.
- qRT-PCR is detecting 0.001 PFU equiv.
- RT-LAMP time-to-positivity is non-quantitative at lower end of sensitivity (and not great at high end either)
- RT-LAMP usually takes <30 minutes.





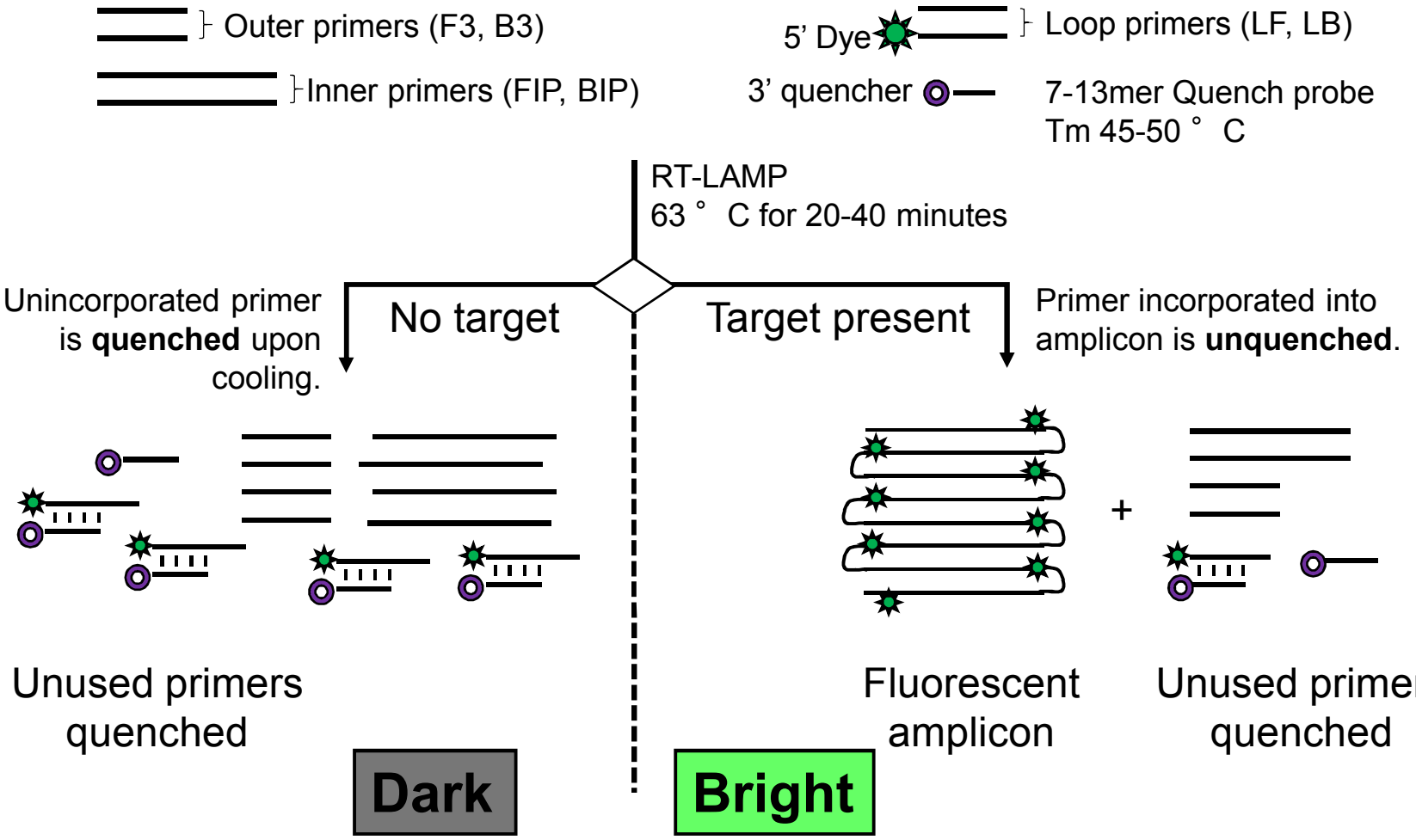


Wheeler *et al*, *PLOS One* 2016

For a deployed LAMP diagnostic:

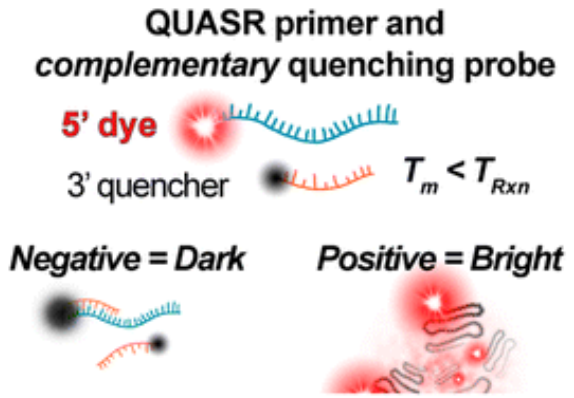
- *Closed tube* detection – don't want to open the tube after the reaction
- *Large discrimination* between positive and negative samples
- *Bright signals* – naked eye or simple detector
- *Target-specific*, vs detecting total DNA
- *Endpoint* is good enough for yes/no answer (LAMP is semi-quantitative at best anyway!)
- Minimize complex instrumentation or operations

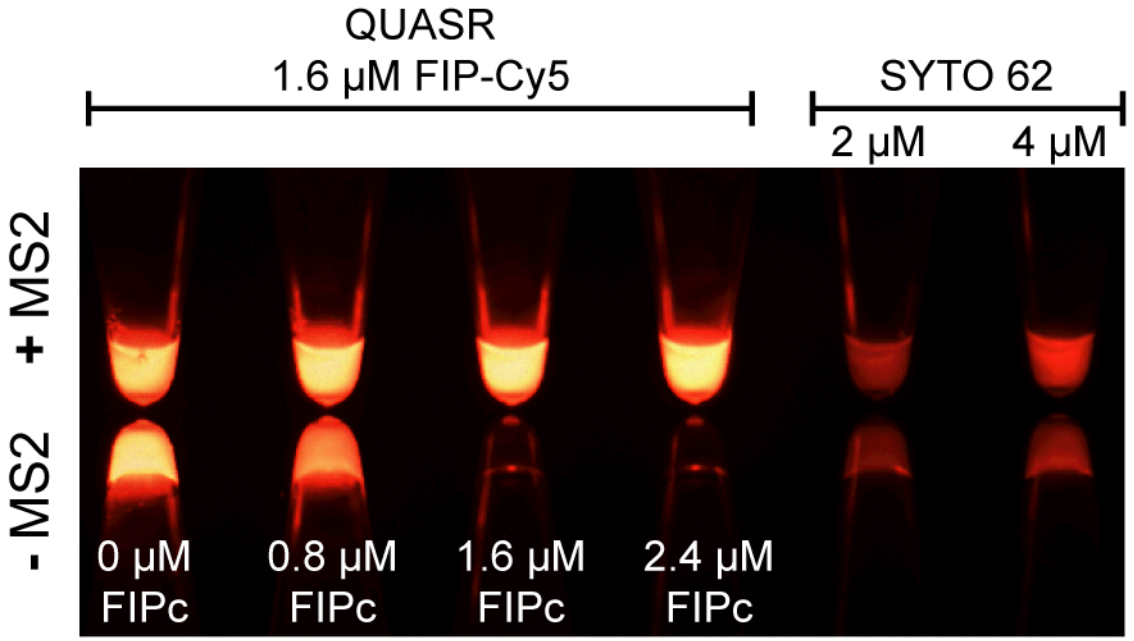
QUASR: Quenching of Unincorporated Amplification Signal Reporters

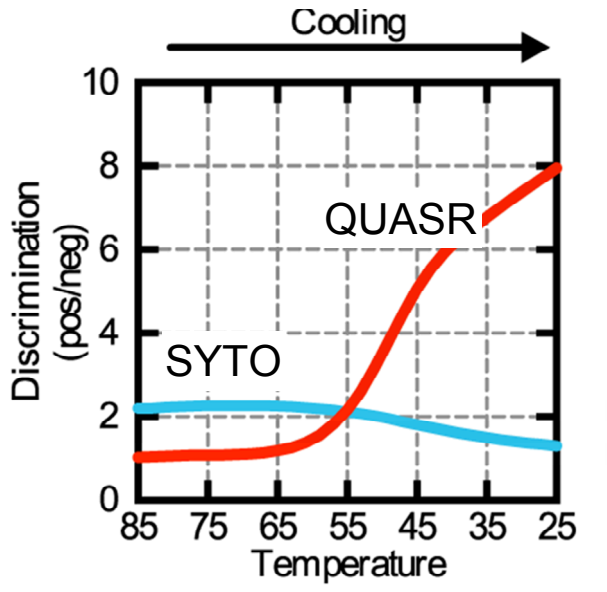


QUASR Proof of Concept (MS2)

- QUASR signal develops as reaction cools below T_m of quench probe
- Contrasts to intercalating dye (*e.g.* SYTO) where discrimination is highest while hot
- Endpoint only, but closed-tube, very bright signals, and target-specific.

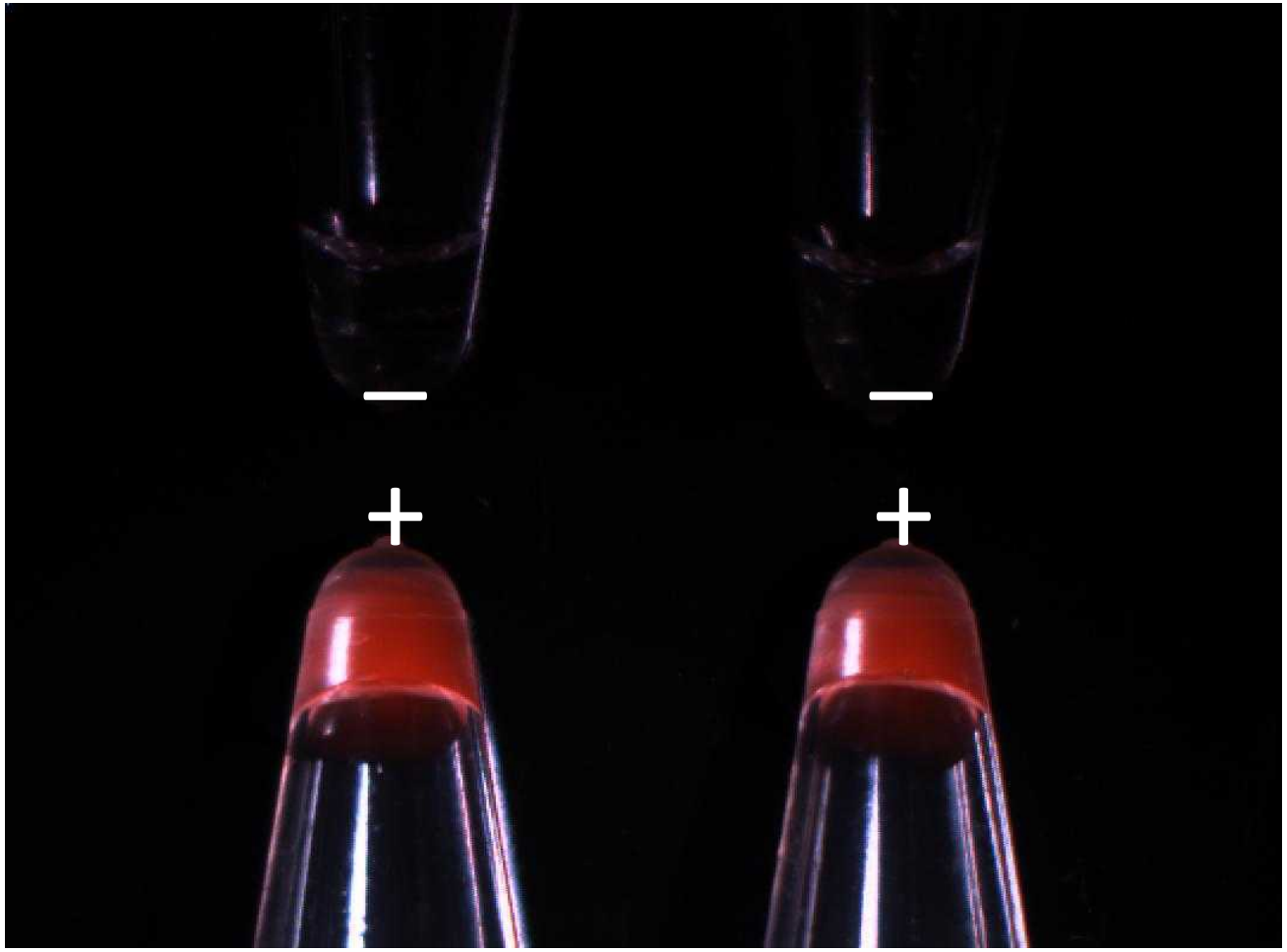






Ball *et al*, *Anal. Chem.* 2016

WNV QUASR assay (ROX label)



Published WNV RT-LAMP primer set from Parida *et al* adapted to QUASR detection

Color photo taken with green LED flashlight and magenta theatre lighting gel as a filter

- ☑ **Closed-tube detection**
- ☑ **Bright endpoint signal**
- ☑ **Large difference between positive and negative**

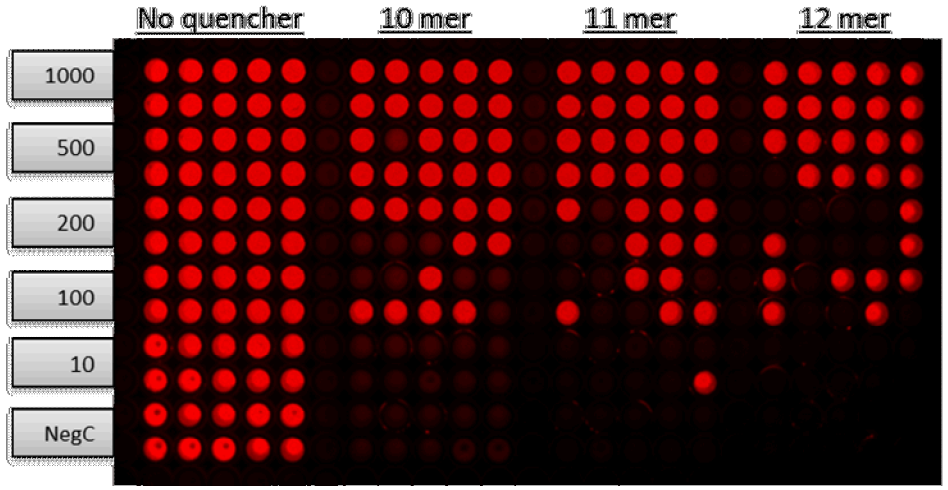
Adapting assays for QUASR

A

Target copies

quencher	1000	500	200	100	10	0
none	10	9	5	8	1	0
10 mer	10	9	6	5	0	0
11 mer	10	9	7	5	1	0
12 mer	10	9	3	6	0	0

B



Quencher optimization for Ebola GP assay

Choose a primer set that already has good properties (*e.g.* fast, high sensitivity, specific, few false positives, and adapt for QUASR)

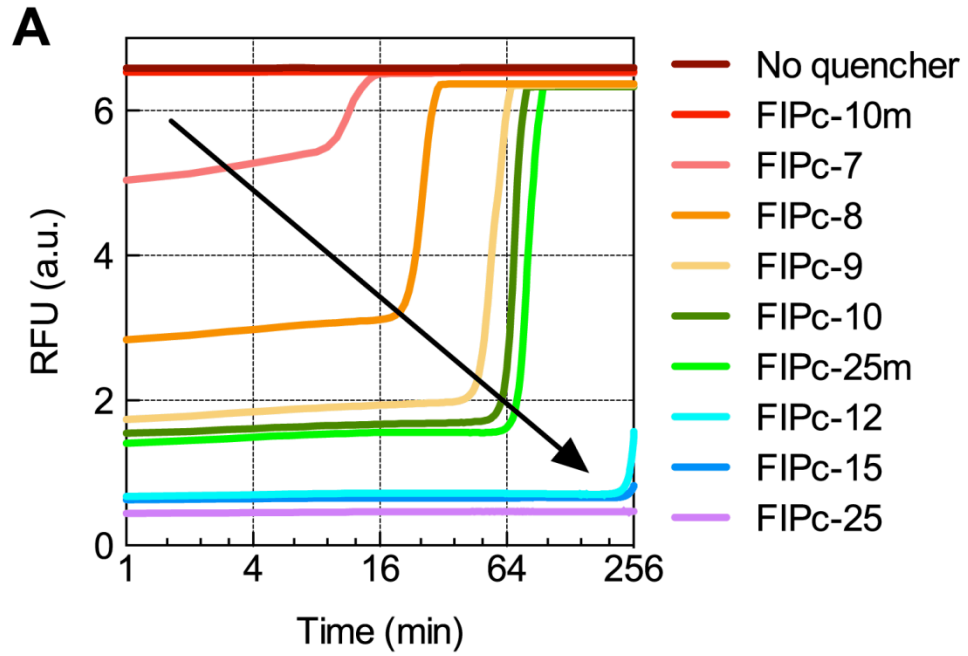
The main design considerations are:

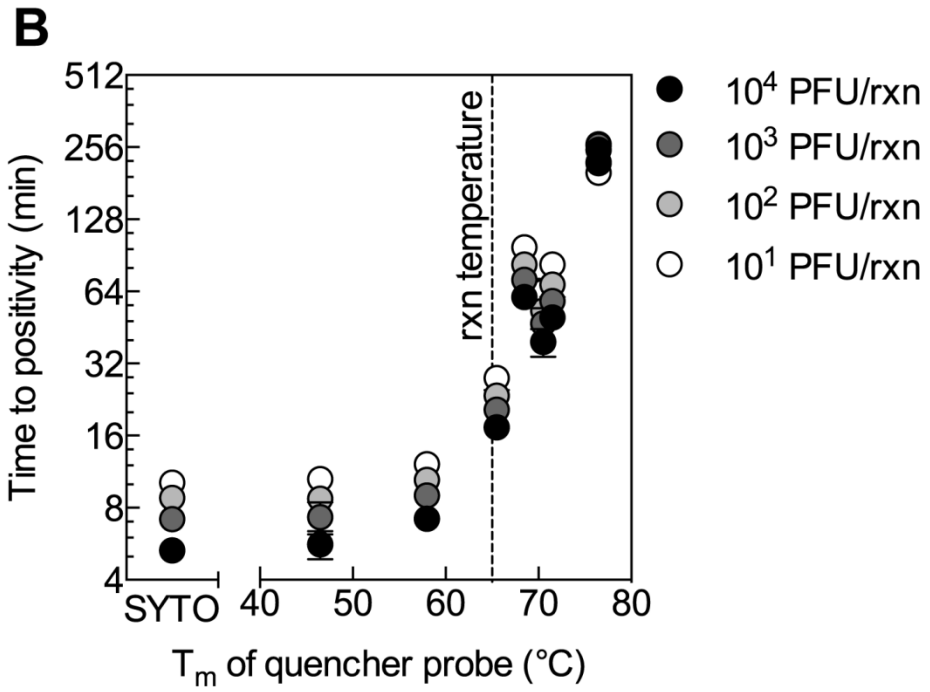
1. Which primer to label – LoopF, LoopB, FIP, and BIP all work
   - FIP, BIP give brighter signal; LoopF/LoopB may have less secondary structure
   - Choose the one with the least-stable hairpin structure at 5' end

2. Length of complementary quench probe
   - aim for Tm 45-50, but also above Tm of most stable hairpin

Optimizing QUASR quench probe design

- As T_m approaches reaction temperature, reaction is inhibited
- If probe T_m is > 50-55 ° C, we can observe probe being displaced in real time, similar to Tanner *et al* "DARQ" *Biotechniques* 2012 but clearly inhibited
- Constraints on lower limit for T_m:
  - Ambient temperature for performing detection
  - Temperature at which labeled primer forms a stable hairpin structure

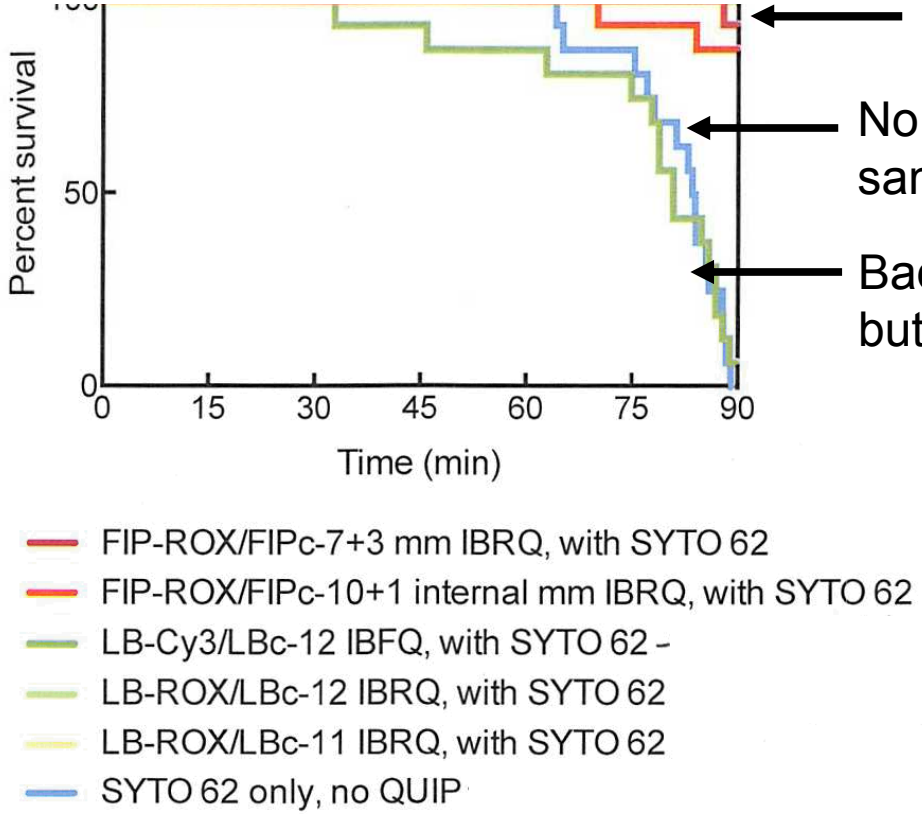




Ball *et al*, *Analytical Chemistry* 2016

QUASR can suppress LAMP false positives

"Survival" of WNV no-template controls (time to appearance of non-specific SYTO 62 signal)



C. Ball, *Analytical Chemistry* 2016

Good QUASR: Spontaneous amplification is suppressed; and not evident in endpoint signal

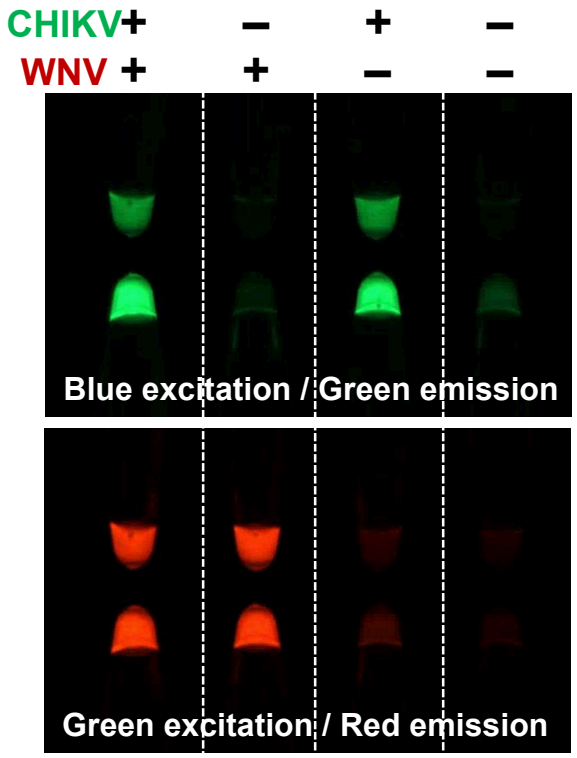
No QUASR: Spontaneous amplification in all samples, and visible with non-specific DNA dye

Bad QUASR? Spontaneous amplification happens, but is not evident in endpoint probe signal (still ok)

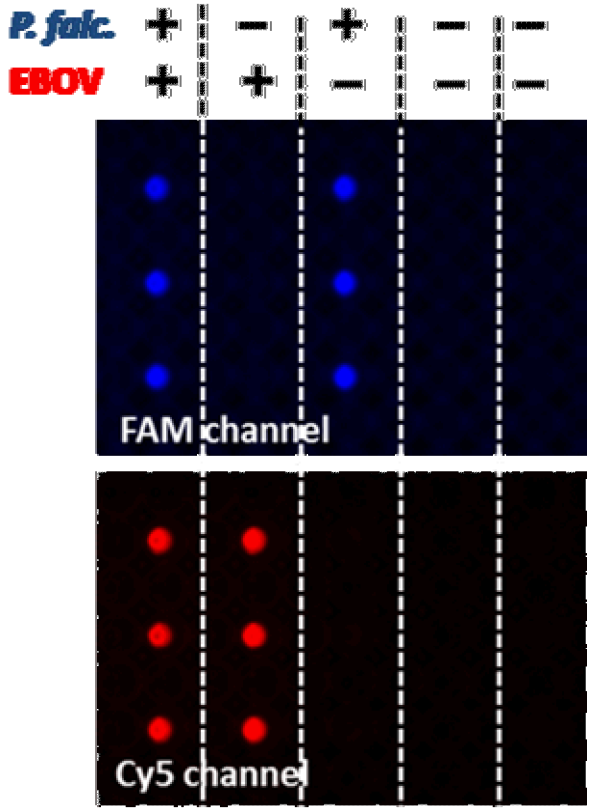
- "False positive" amplification usually occurs >50 minutes, so we define a cutoff of 30-40 minutes
- Even if a sample shows false positive with the SYTO dye, the QUASR signal is usually still negative
- *e.g.* 1/197 FP for QUASR, *vs* 67/145 FP for SYTO, in 90-minute extended rxns)

Multiplexing RT-LAMP with QUASR

(A) Chikungunya virus + West Nile virus



(B) *Plasmodium falciparum* + Ebola virus



Yooli Light

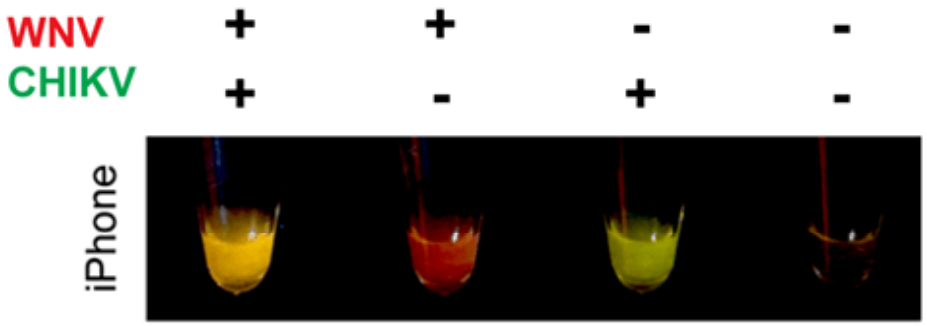
☑ **Target specific** ☑ **Muliplexable**

LAMP historically is hard to multiplex, because the polymerase lacks the 5'-3' exonuclease activity that enables Taqman assays.

Smart Phone as a Diagnostic Platform

- Ubiquitous even in developing world
- Even older model smart phones have powerful processors and good cameras
- Solutions are common for “microgrid” charging even in remote places without reliable electricity
- QUASR signals are bright enough to visualize with a smart phone camera.
- Can we leverage smart phone to create a portable assay platform for Zika virus?

Visualization of duplex QUASR with an LED flashlight, iPhone, and colored plastic filter



Ball *et al*, *Anal. Chem.* 2016

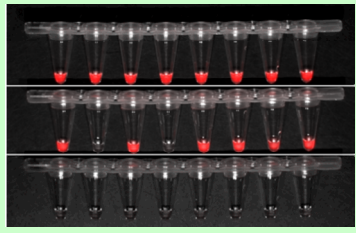
Phone charging kiosks in Uganda

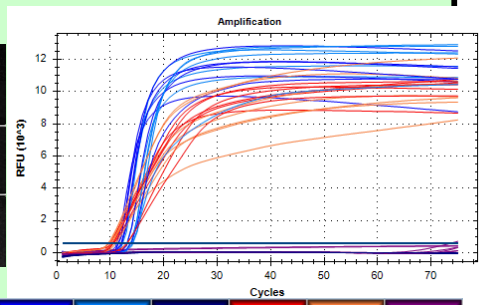
Jenny Matthews / Alamy; in *Nature* 2014

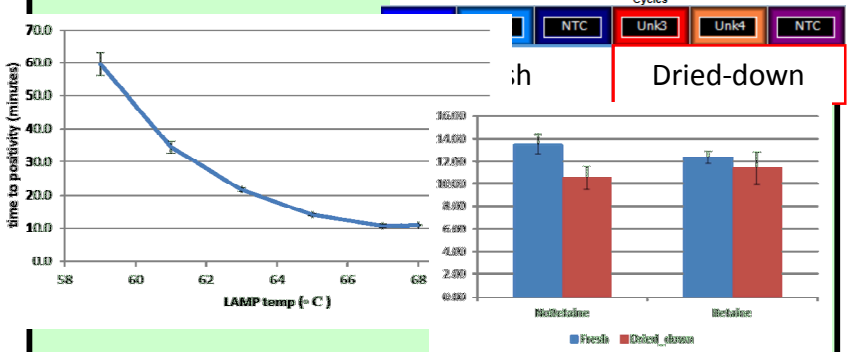
Wikipedia/Ken Banks

Zika Assay By Smart Phone

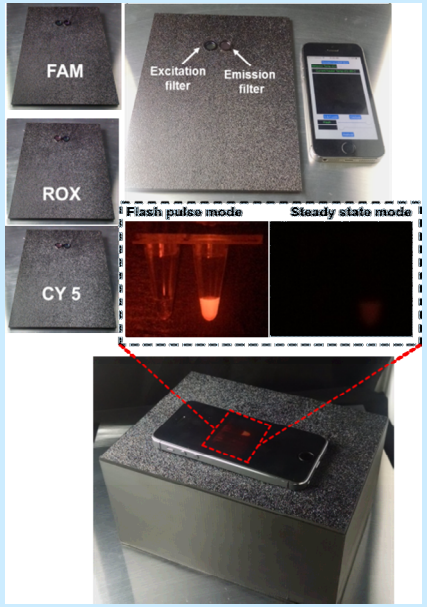
Design and evaluation of new ZIKA QUASR assay

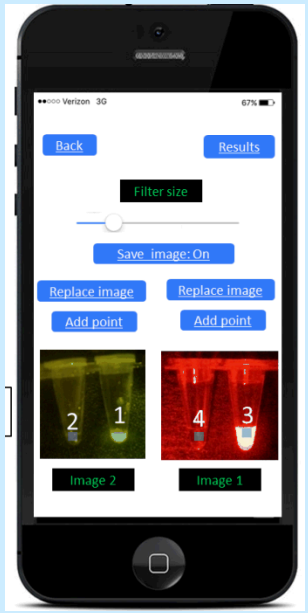






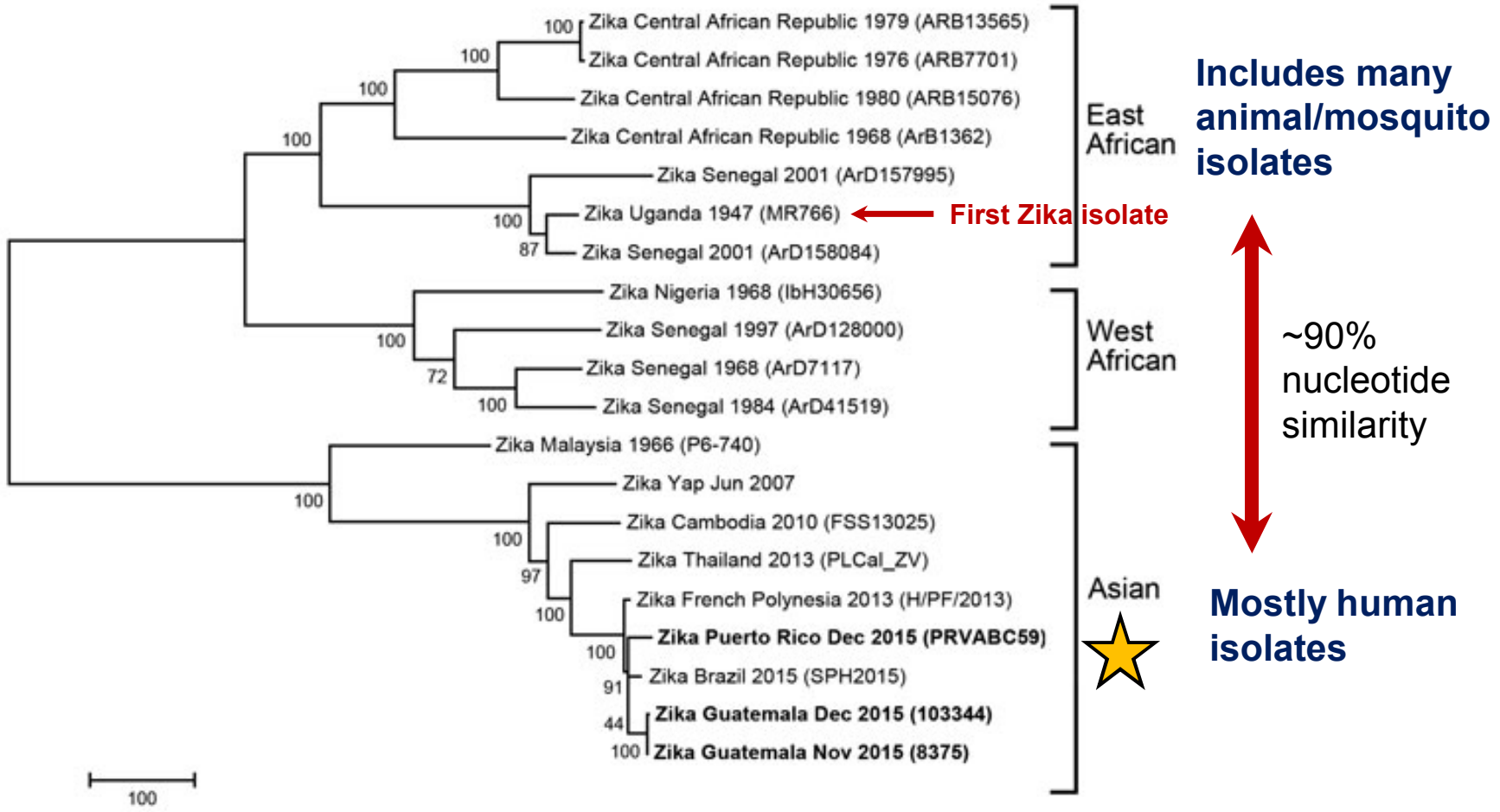
Design and testing of portable Smart Phone assay platform





Zika assay on Smart Phone platform

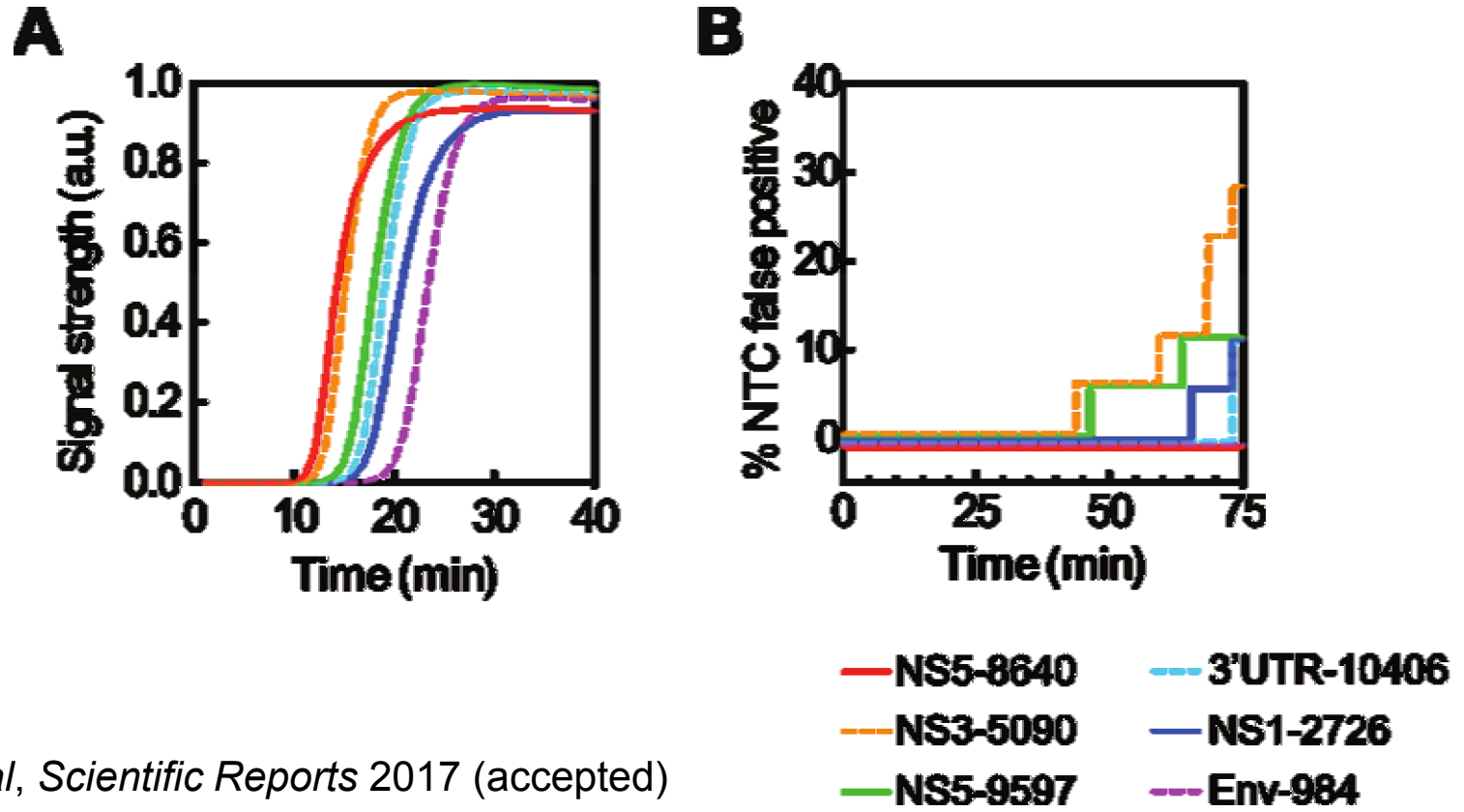
Primer Design for ZIKA



Lanciotti *et al*, Emerging Infectious Diseases 2016

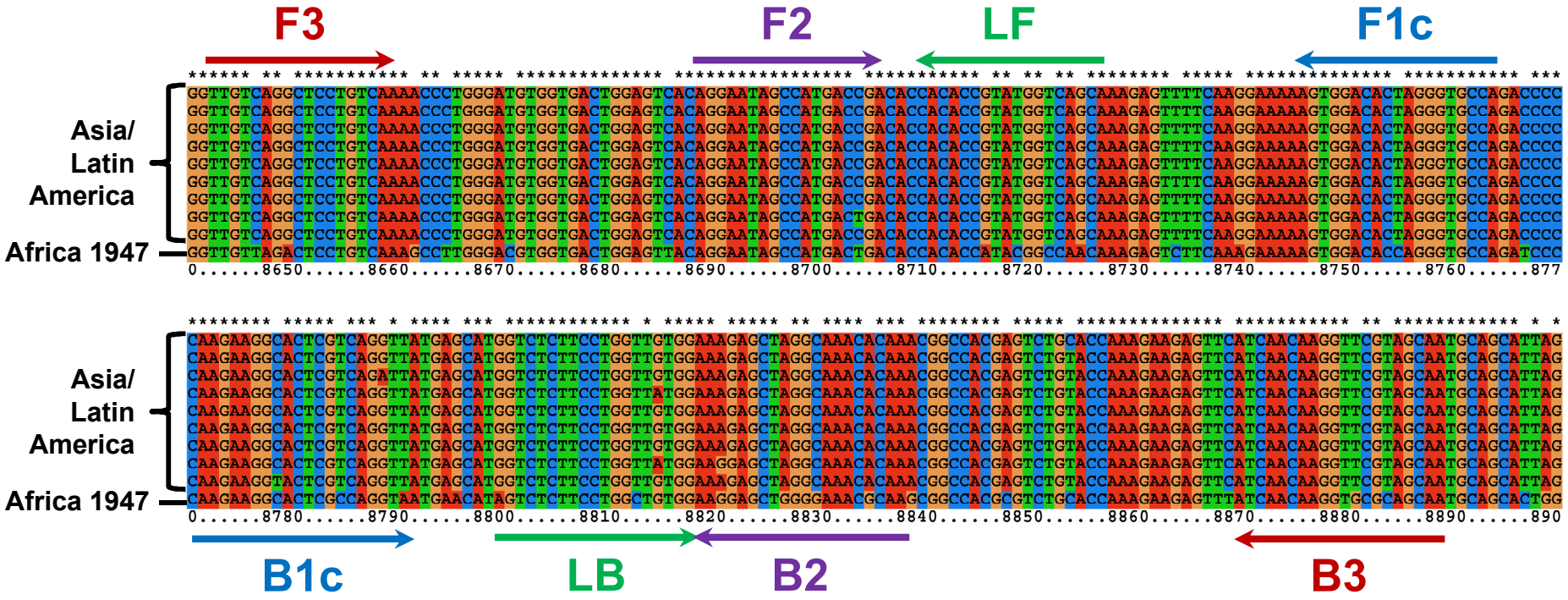
RT-LAMP Primer set testing

- Optimize for speed of amplification and low rate of false positives with nonspecific SYTO dye
- Then convert to QUASR set for further evaluation



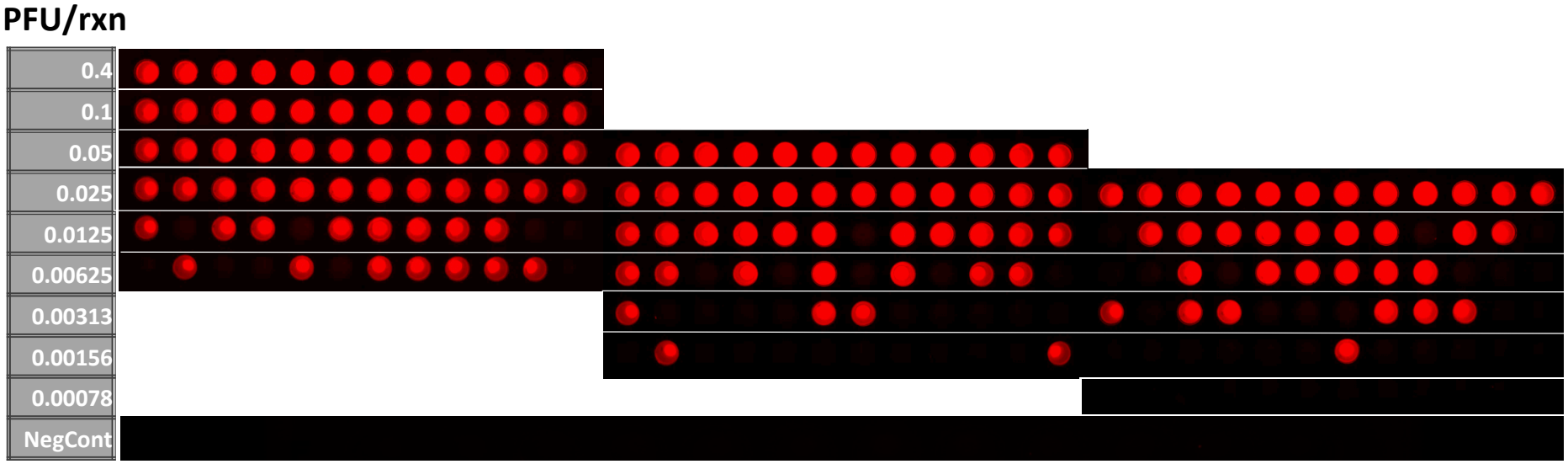
Priye *et al*, *Scientific Reports* 2017 (accepted)

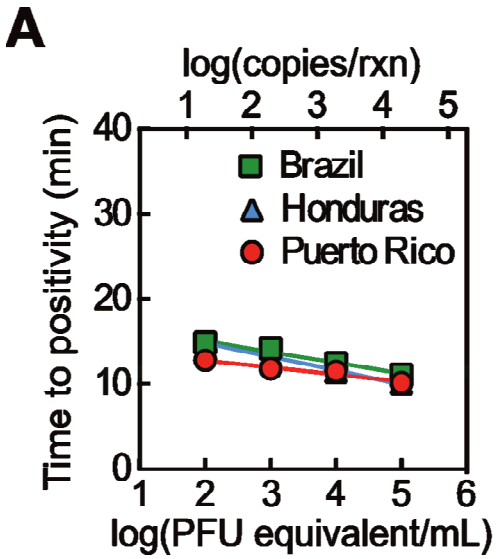
LAMP primers targeting NS5 gene

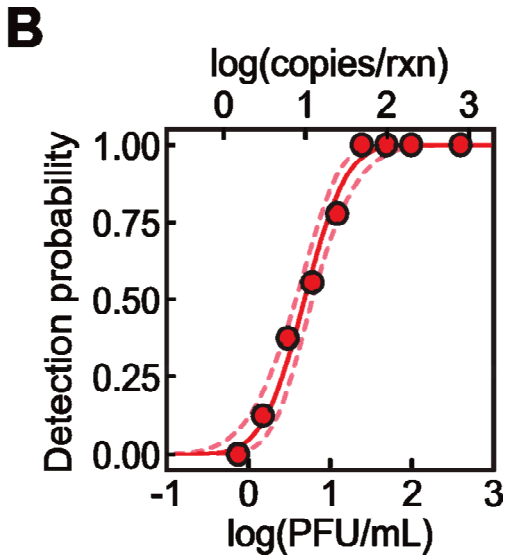


- Set targeting NS5 (RNA-dependent RNA polymerase) provided best sensitivity and reaction speed
- Good match for Asian lineage but 15 mismatches to Africa 1947 (broad range LAMP primers are hard for RNA viruses)!
- No cross-reactivity with panel of related viruses

Zika NS5 LAMP Sensitivity







Sensitivity testing (10-30 replicates) with *intact virus* spiked into reaction buffer (no lysis/extraction)

<u>Detection probabilities</u>

95% - 22 PFU/mL (5×10^4 copies/mL)

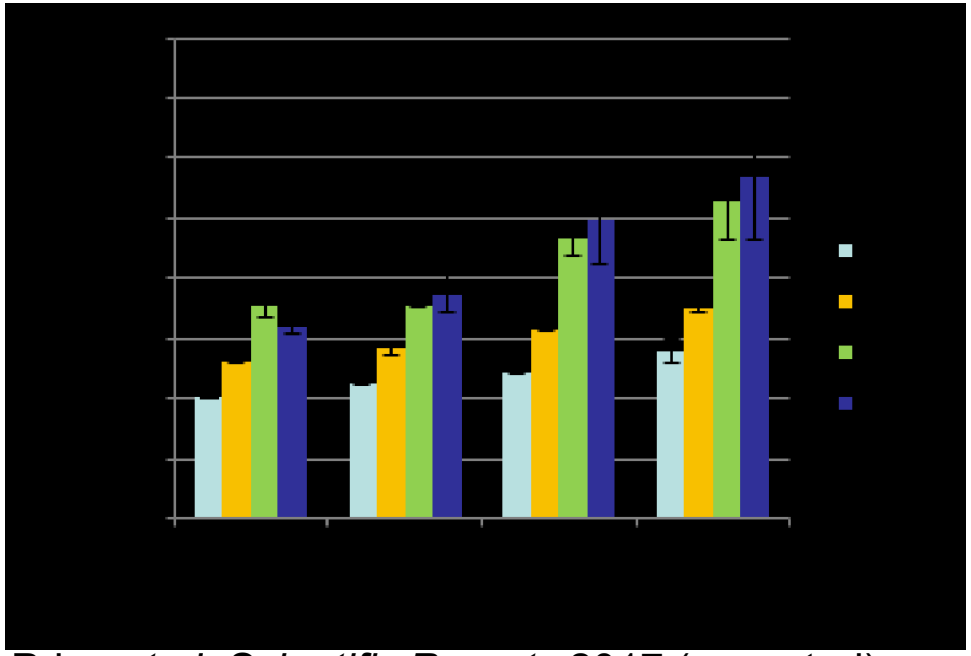
50% - 4.9 PFU/mL (10^4 copies/mL)

Priye *et al*, *Scientific Reports* 2017 (accepted)

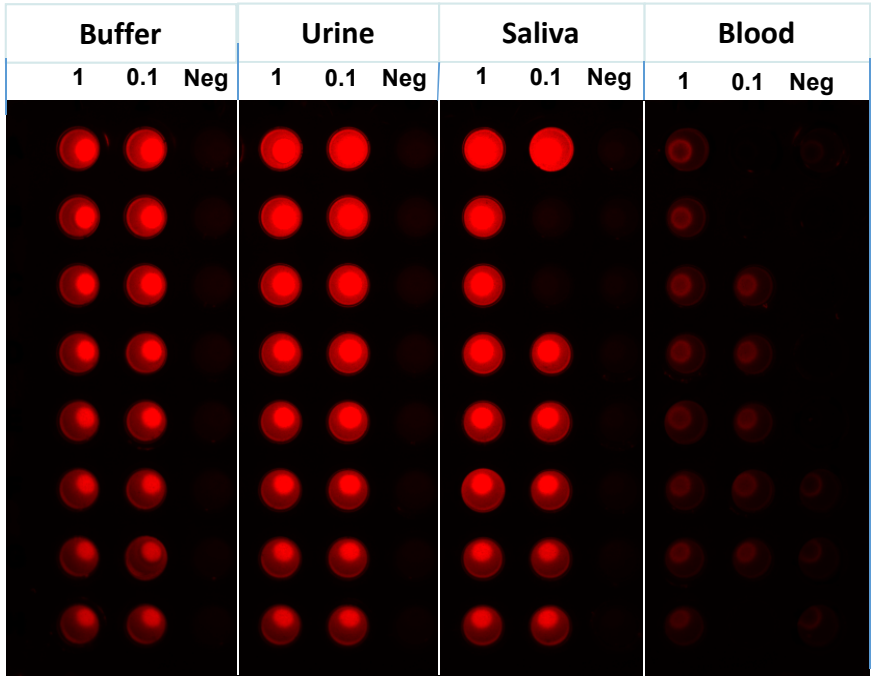
Zika LAMP in clinical matrices

- Virus spiked directly into human blood, saliva, or urine
- Spiked samples added (1/10 dilution) into LAMP mix
- Good performance in urine
- Reactions slow down somewhat in saliva and blood, slight dropoff in rate of positive detection at 0.1 PFU

Time to positivity



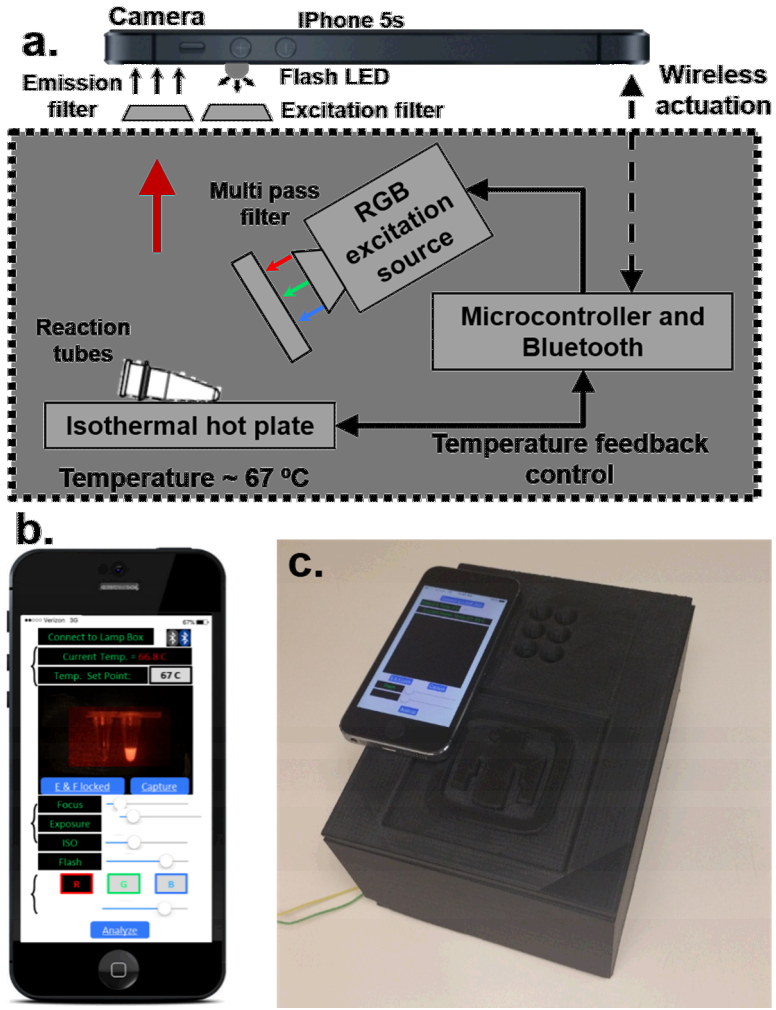
QUASR End-point



Priye *et al*, *Scientific Reports* 2017 (accepted)

Smart Phone platform overview

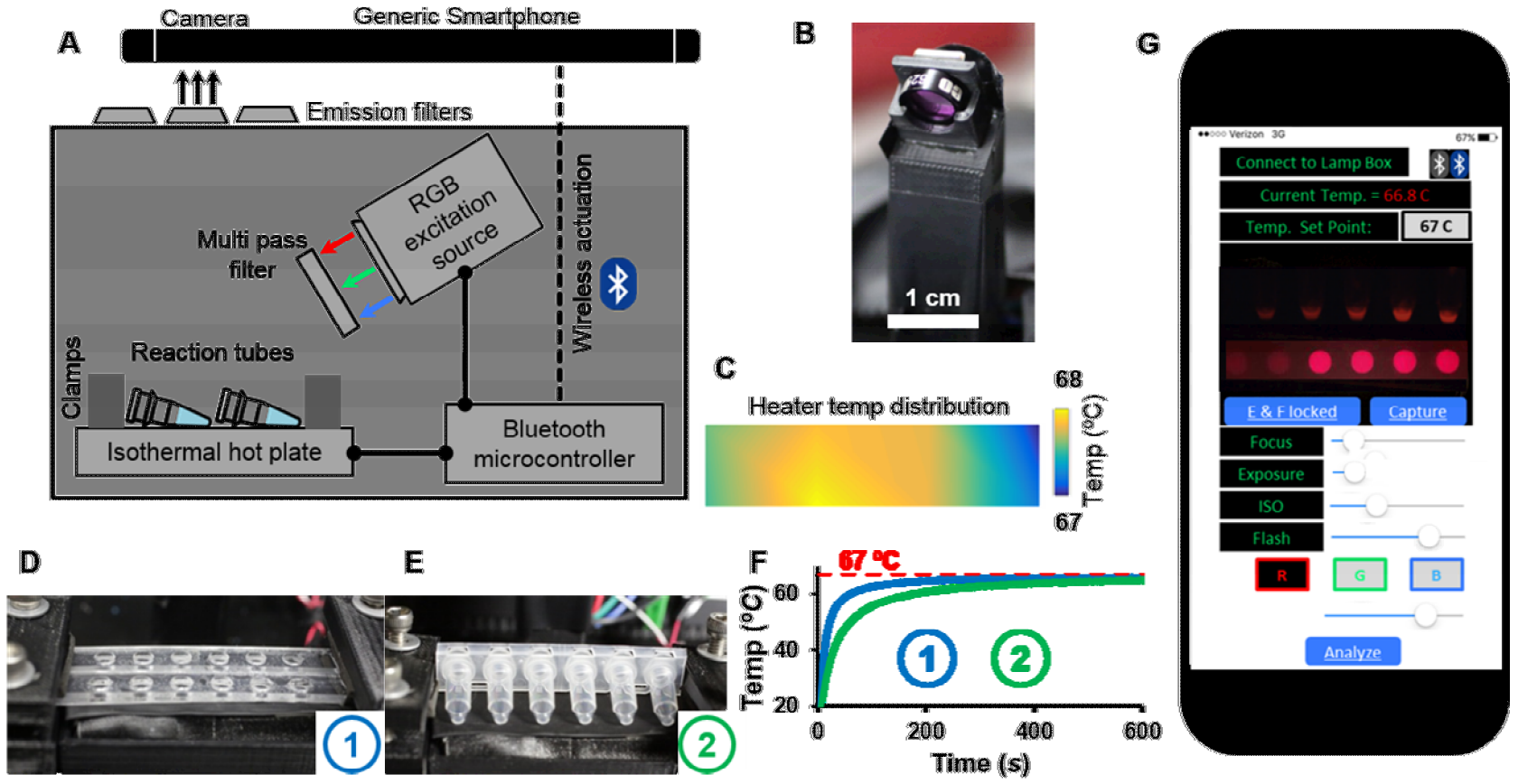
- Handheld box contains heater, optics, and Bluetooth enabled microcontroller
- Compatible with iPhone and Android phones
- Smart phone app “front end” controls heater, timing, and fluorescence image acquisition
- Heater accepts a variety of formats: PCR tubes, microwells, or planar chips
- Hardware costs: about $50 with plastic filters; $500 with high quality coated glass filters, plus phone
  - Compare to $18-20k for portable isothermal fluorimeter such as Genie III.



Priye *et al*, *Scientific Reports* 2017 (accepted)

LAMP in Smart Phone box

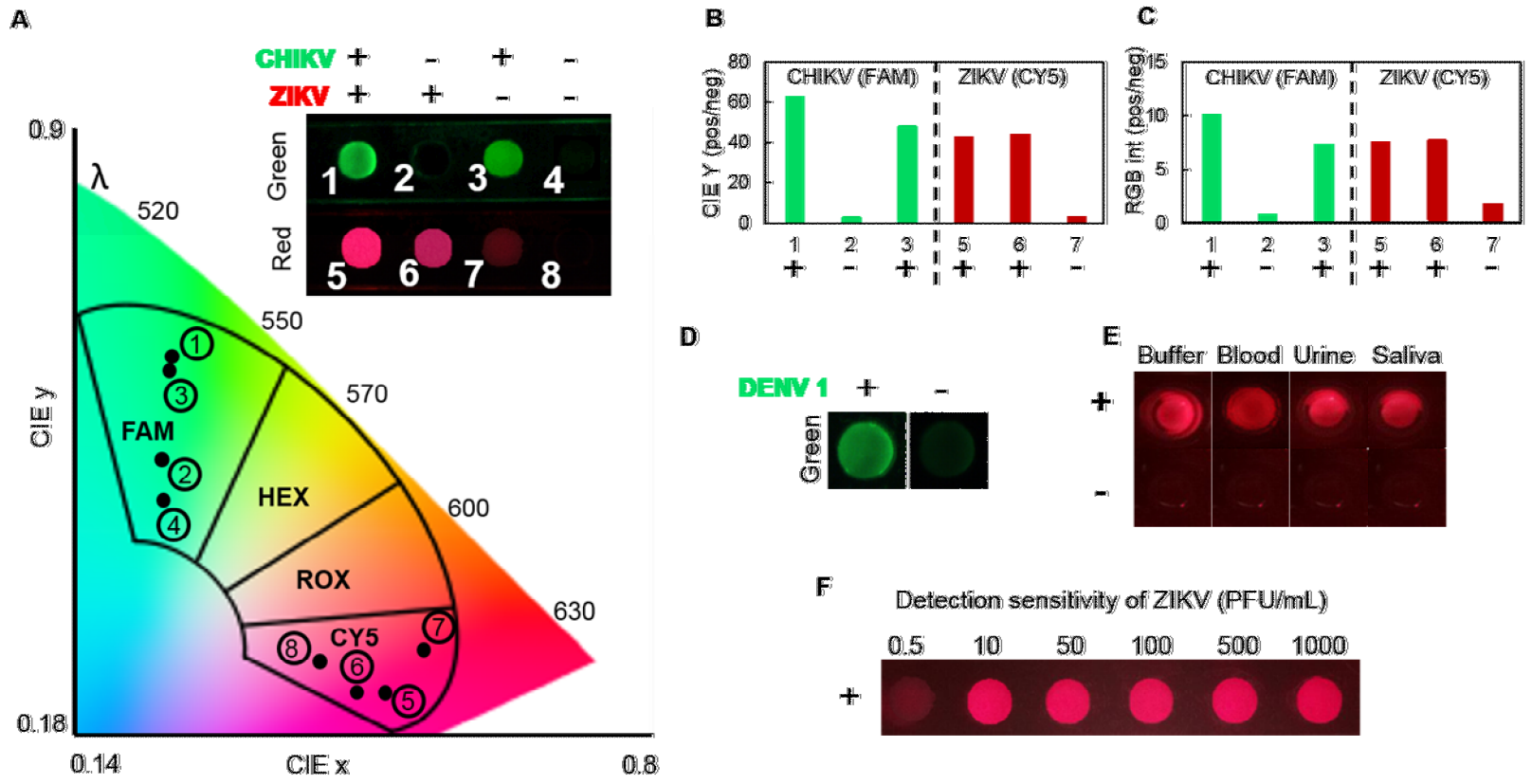
- Works with standard PCR tubes, or with custom-fabricated planar microwells, or even inside of PCR tube cap strips sealed with Microseal B film.



Priye *et al*, *Scientific Reports* 2017 (accepted)

Smart Phone image analysis

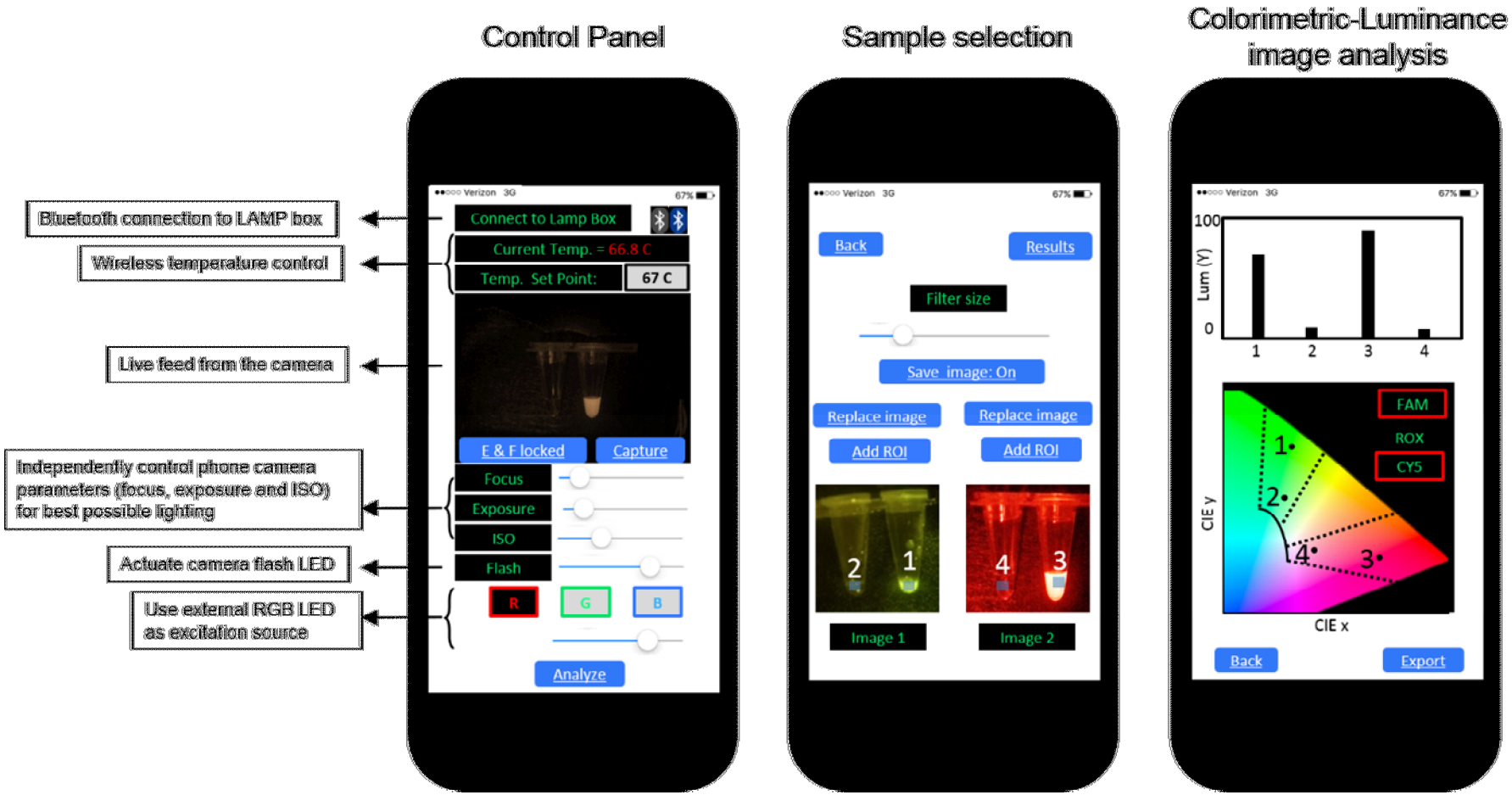
Phone app maps multicolor fluorescence images onto chromaticity-luminance (CIE xyY) color space to allow automated assay scoring



Example of 2-color analysis for ZIKV/CHIKV duplex, with separate one-pot RT-LAMP assay for DENV.

Priye *et al*, *Scientific Reports* 2017 (accepted)

LAMP2Go App



Versions for iPhone and Android
Older model phones ok (*e.g.* <$100 HTC Desire)

Relevant Publications

S. Wheeler *et al*, Surveillance for Western Equine Encephalitis, St. Louis Encephalitis, and West Nile Viruses Using Reverse Transcription Loop-Mediated Isothermal Amplification, *PLoS One* 2016 (RT-LAMP with real-time monitoring, melt curve multiplexing, no QUASR)

C. Ball *et al*, Quenching of Unincorporated Amplification Signal Reporters in Reverse-Transcription Loop-Mediated Isothermal Amplification Enabling Bright, Single-Step, Closed-Tube, and Multiplexed Detection of RNA Viruses, *Analytical Chemistry* 2016 (description of QUASR method)

C. Ball *et al,* A simple check valve for microfluidic point of care diagnostics, *Lab on a Chip* 2016 (QUASR employed in microfluidic device)

A. Priye *et al*, A smartphone-based diagnostic platform for rapid detection of Zika, chikungunya, and dengue viruses, *Scientific Reports* 2017 (accepted)

N. Tanner *et al*, Simultaneous multiple target detection in real-time loop-mediated isothermal amplification, *Biotechniques* 2012 (DARQ technique, with real-time displacement of ‘probes’, partial inspiration for QUASR endpoint technique)

D. Rudolph *et al*, Detection of Acute HIV-1 Infection by RT-LAMP, *PLoS One* 2015 (uses a technique that resembles either QUASR or DARQ)